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Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions

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Abstract

This paper examined the effect of several pyridinium and imidazolium-based ionic liquids (ILs) on the protease stability in aqueous solutions. In general, the enzyme was found quite active at low concentrations of hydrophilic ILs. In aqueous environment, the enzyme was stabilized by the kosmotropic anions (such as CF_3COO^- and CH_3COO^-) and chaotropic cations (such as $[BuPy]^+$ and $[EMIM]^+$), but was destabilized by chaotropic anions (such as tosylate and BF_4^-) and kosmotropic cations (such as $[BMIM]^+$).

Keywords: Ionic liquid; Enzyme; Activity; Stability; Kosmotrope; Chaotrope; Kosmotropicity

1. Introduction

In aqueous solutions, the effect of ions on the enzyme activity could be related to the Hofmeister lyotropic series [1]. Small or multiply-charged ions with high charge density are called kosmotropes ('order-maker') because they strongly order water molecules. These ions include citrate, CH_3COO^-, SO_4^{2-}, HPO_4^{2-}, Mg^{2+}, Ca^{2+}, Li^+, Na^+, H^+, OH^-, and more. The kosmotropes have stronger interactions with water molecules than water with

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itself and thus capable of breaking water–water hydrogen bonds. But the overall entropy is lower because the water molecules are highly organized near the cation. For a given charge density, anions are more strongly hydrated than cations [2]. A study revealed that the degree of activation of *Subtilisin* Carlsberg is 2800-fold in CH_3COONa solutions than in the salt-free solution due to kosmotropic acetate ions [3]. The contrary concept of kosmotrope is chaotrope ('disorder-maker'). The chaotropic ions include those large and singly-charged ions such as SCN^-, $H_2PO_4^-$, HSO_4^-, HCO_3^-, I^-, NO_3^-, BF_4^-, NH_4^+, Cs^+, K^+, $(NH_2)_3C^+$ (guanidinium), $(CH_3)_4N^+$ (tetramethylammonium), etc. The chaotropes have weaker interactions with water molecules than water with itself. The hydrogen bonding between water molecules is less broken in the immediate vicinity of ionic chaotropes than ionic kosmotropes. But the overall water structure is more broken by the chaotrope. As a result, macromolecules including enzymes have more structural freedom for extension and denaturation [2,4]. For example, two important chaotropes (guanidinium and thiocyanate ions) were found poorly hydrated, allowing them to preferentially interact with the protein rather than the water [5].

It has been known that strong kosmotropic anions stabilize proteins while strong kosmotropic cations destabilize them (Fig. 1) [10–12]. Therefore, an optimal stabilization of enzymes or other biological macromolecules is usually achieved through the use of salts containing kosmotropic anions and chaotropic cations [1,6,10,12]. Warren and co-workers [13,14] noticed that neutral salts inhibited the enzyme activity in an order of increasing effectiveness for anions: $CH_3COO^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < ClO_4^-$ (decreasing kosmotropicity), and for cations: $(CH_3)_4N^+ < Cs^+ < K^+ < Na^+ < Li^+$ (increasing kosmotropicity). Our recent review exemplified the effect of ion kosmotropicity on the enzyme activity and stability [1]. The ion kosmotropicity could be quantified by many thermodynamic parameters such as viscosity *B*-coefficients, structural entropies, structural volumes, NMR *B*′-coefficients, and ion mobility. For example, kosmotropic ions usually have larger viscosity *B*-coefficients than chaotropic ones (Fig. 1). Recently, we further discussed the availability of these parameters for quantifying various ions especially those contained in ionic liquids [9].

As a new generation of organic salts, ionic liquids (ILs) have demonstrated their exciting potentials as novel solvents for various reactions including biocatalysis as discussed in recent reviews [15–20]. A general finding from previous studies indicated that enzymes could maintain their activity and stability in certain ionic media. However, not all ILs are suitable for an enzyme in a particular biocatalysis reaction. For instance, free lipase (*Candida rugosa*) was found not active in pure hydrophilic ILs (those have anions of NO_3^-, CH_3COO^-, CF_3COO^-, $CF_3SO_3^-$, and $CH_3SO_3^-$), but active in a hydrophobic IL based on the anion PF_6^- [21]. There is no simple answer to whether an enzyme is active in a certain IL because the enzyme

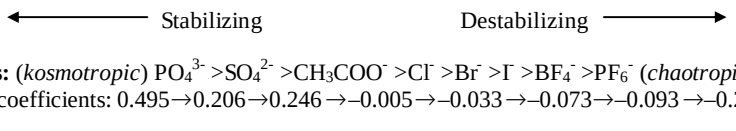


Fig. 1. The Hofmeister series of the ion effect on protein stability [6,7] (The *B*-coefficients are from Marcus' collection [8]; $(CH_3)_4N^+$ is a chaotrope, and its unusually large *B*-coefficient is due to the hydrophobic hydration [9]).

activity depends on the enzyme–medium–substrate relationship. However, several factors of ILs, e.g., polarity [15], hydrogen-bond basicity [22,23] and anion nucleophilicity [21], have shown strong influences on the activity and stability of enzymes.

Currently, most ILs used in biocatalysis are hydrophobic types of PF_6^- and $(CF_3SO_2)_2N^-$ salts [15–18]. Hydrophobic solvents could be superior to hydrophilic types because the latter could remove internally bound water from the enzyme [24]. The enzymes were practically suspended rather than dissolved in the hydrophobic media. The resulting heterogeneous environment prevented the enzyme from denaturation by the high ionic strength. For example, Basso et al. [25] observed that in order to maintain the water activity (a_w) compatible with the activity ($a_w > 0.70$) of immobilized penicillin G amidase (PGA-450 containing 35% water), 1% water was sufficient for hydrophobic [BMIM][PF_6] and [OMIM][PF_6], while 10, 5, 20, and 20% were needed by hydrophilic [BMIM][BF_4], [OMIM][BF_4], [BMIM][CH_3SO_4], and [OMIM][CH_3SO_4], respectively. Meanwhile, some enzymes were also found active and stable in anhydrous hydrophilic ILs as they were in anhydrous organic solvents [26–28].

However, there are only a few studies concerning the enzyme activity in water-containing hydrophilic ILs (Table 1). When a significant amount of water is involved, the hydrophilic ILs dissociate into individual ions. Therefore, the Hofmeister series might be applicable to the IL effect on the enzyme activity. For example, Hinckley et al. [29] noticed that the enzyme activity of laccase C from *Trametes* sp. decreased dramatically with the increase of concentration of 4-methyl-*N*-butylpyridinium tetrafluoroborate ([4-

Table 1
Examples of biocatalysis in hydrophilic ILs containing water

IL (H_2O, % v/v)	Biocatalyst (activity)	Reaction	Reference
[$EtNH_3$][NO_3] (>20%)	Alkaline phosphatase (medium)	Hydrolysis of *p*-nitrophenyl phosphate	[31]
[EMIM][BF_4] (2%) [BMIM][BF_4] (2%)	α-Chymotrypsin (low)	Transesterification	[32]
[MMIM][$MeSO_4$] (75%) [BMIM][BF_4] (75%)	Formate dehydrogenase (high) Formate dehydrogenase (low)	Regeneration of NADH	[33]
[EMIM][BF_4] (2%)	*Candida antarctica* lipase B (high)	Transesterification	[34]
[BMIM][BF_4] (50%)	β-Galactosidase and Subtilisin protease Savinase (low)	Esterification	[35]
[EtPy][CF_3COO] (15%)	*Bacillus licheniformis* protease (relatively high)	Kinetic resolution of amino acid esters	[36,37]
[4-MBPy][BF_4] (25–100%)	Laccase C from *Trametes* sp. (medium)	Oxidation of syringaldazine	[29]
[BMIM][BF_4], [OMIM][BF_4], [BMIM][CH_3SO_4], [OMIM][CH_3SO_4] (5–20%)	Immobilized penicillin G amidase (PGA-450 containing 35% water) (medium to high)	Synthesis of the amide of L-phenylglycine methyl ester with methyl phenglacetate	[25]
[BMIM][BF_4], [BMIM]Cl (75–95%)	Horseradish peroxidase (HRP) (medium to high)	Activity assay of H_2O_2, phenol and 4-aminoantipyrine in 0.1 M phosphate buffer	[30]

Note. EMIM, 1-ethyl-3-methylimidazolium; MMIM, 1,3-dimethylimidazolium; BMIM, 1-butyl-3-methylimidazolium; EtPy, *N*-ethylpyridinium; 4-MBPy, 4-methyl-*N*-butylpyridinium; OMIM, 1-octyl-3-methylimidazolium.

MBPy][BF_4]): 10% IL reduced half of the activity and 20% IL reduced the activity more than 9-fold. The high chaotropicity of BF_4^- ions might be an important factor causing the deactivation. Another example [30] indicated that the horseradish peroxidase (HRP) maintained its high activity in [BMIM][BF_4] or [BMIM]Cl solutions up to 20% (v/v or w/v) (activity assay in 0.1 M phosphate buffer). Interestingly, the presence of 5–10% (v/v) [BMIM][BF_4] seemed to improve the HRP thermal stability.

This aim of this work was to investigate the effect of ions (dissociated from ILs) on the protease activity and stability in aqueous environment. The correlation between the enzyme stability and ion kosmotropicity was further related to the Hofmeister series.

2. Materials and methods

2.1. Materials and enzymes

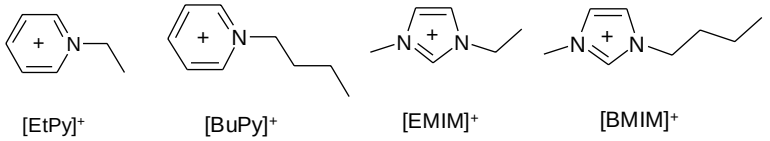
Amano protease P6 was generously provided by Amano Enzyme USA. This enzyme in the powder form was produced from a selected strain of *Aspergillus melleus* and possesses a high proteolytic activity at pH 5–9. *N*-Ethylpyridinium bromide ([EtPy]Br) and *N*-*n*-butylpyridinium chloride ([BuPy]Cl) were obtained from the Alfa Aesar. 1-Ethyl-3-methylimidazolium bromide ([EMIM]Br) and 1-butyl-3-methylimidazolium bromide ([BMIM]Br), 1-ethyl-3-methylimidazolium tosylate ([EMIM][OTs]), Novozymes alcalase (*Bacillus licheniformis* protease, P4860), PPL (porcine pancreas lipase, L3126), N_α-*p*-tosyl-L-arginine methyl ester hydrochloride, Nile Red, and other reagents were purchased from Sigma–Aldrich.

2.2. IL preparations

ILs were prepared using the silver metathesis method illustrated in literatures [38,39]. Charcoal was used to remove color and impurities from crude ILs effectively [40]. The silica-gel column was further used to remove trace impurities. Water was evaporated through a rotary evaporator under vacuum at 50 °C. All ILs prepared are slightly viscous and colorless liquids. The structures of their cations were illustrated in Scheme 1. ^{1}H NMR, FT-IR, and HPLC data confirmed that the prepared ILs are free of measurable impurities including water.

2.3. Measurements of activity and stability

The general method is a modification of a literature method for examining the protease activity in organic solvents [41]. Enzyme incubation media were mixtures of ILs (or organic solvent) and water. Five milligrams of the enzyme was placed in a 6 mL vial. Into this viral, at time zero, 1 mL of a particular incubation medium was added. The incubation vial was maintained at 25 ± 1 °C. Except in ILs based on [BF_4]$^-$, the protease P6 and alcalase were



Scheme 1. Structures of cations in ILs.

very soluble in all media. The PPL was insoluble and suspended in aqueous solutions. The *initial activity* (*or 100% activity*) of an enzyme was taken to be 1 min after the addition of the solvent for a complete dissolution of the enzyme. The pH of all media was 7 except 3–4 in $[BF_4]^-$ based ILs. The enzyme was incubated in IL solutions without using buffer because ions in buffer (such as phosphate) could considerably affect the enzyme activity as well [1].

To examine the enzyme activity, 0.1 mL of the incubated enzyme solution was added into 1 mL of 0.2 M phosphate buffer (pH 8.0) containing 10 mM *N*-(*p*-toluenesulfonyl)-L-arginine methyl ester (TAME). The reaction mixture maintained pH of 8.0 in this buffer solution. The progress of TAME hydrolysis was analyzed by the LC-10AT Schimadzu HPLC (Schimadzu Premier C18 column with a dimension of 150 mm × 4.6 mm and particle size of 5 μm, flow rate at 1.0 mL/min, water/MeOH ratio of 80/20, and wavelength at 247 nm). Upon the sample injection into HPLC, there is an immediate separation of the enzyme from TAME by the column preventing the further reaction. All experiments were run in duplicates. The initial reaction rate was calculated from the average TAME conversion in 2 min (minimum allowable time for the HPLC injection).

2.4. Measurements of IL polarity

The polarities of ILs using Nile Red were measured based on a literature method [42].

3. Results and discussion

3.1. Effect of IL concentration on the enzyme stability

The enzyme activity is salt-concentration dependent. As shown in Fig. 2, the protease exhibited higher stabilities in 0.7 M aqueous solutions of [BuPy][CF_3COO] and

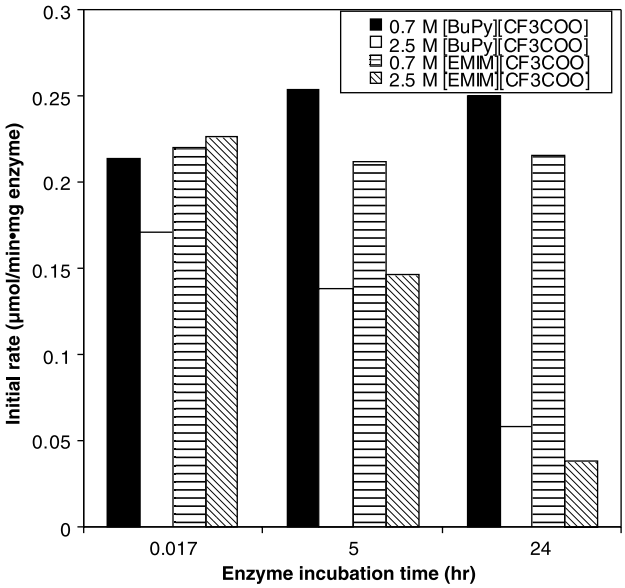


Fig. 2. Effect of IL concentration on the enzyme stability.

[EMIM][CF_3COO] than in their 2.5 M solutions, respectively. This was probably because the high ionic strength inhibits the enzyme activity as traditional inorganic salts do. For this reason, we used a low concentration of ILs (0.7 M) for the following study of the enzyme activity.

3.2. Effect of anions on the enzyme stability

The IL structures impose a strong influence on the enzyme activity. The protease exhibited no measurable activities when it was incubated in 0.7 M solutions of all $[BF_4]^-$ based ILs (namely [EtPy][BF_4], [BuPy][BF_4], [EMIM][BF_4], and [BMIM][BF_4]). The protease stabilities in other pyridinium and imidazolium based ILs (0.7 M) were compared with organic solvents in Figs. 3 and 4, respectively. The enzyme activity was not well maintained in aqueous solutions of organic solvents such as ethanol and acetonitrile. It is well-known polar organic solvents tend to deactivate enzymes [38,43]. The organic solvents used (e.g., ethanol and acetonitrile) are hydrophilic, and the enzyme denatured faster in the hydrophilic organic solvents than in hydrophobic ones [24]. Water provided a relatively suitable environment for the protease to stay active, but it is not always the choice of solvents because substrates and enzymes may not be soluble in water, and non-enzymatic reactions and autolysis may occur in pure water as well [44].

The IL anions have a significant impact on the enzyme activity. Based on Figs. 3 and 4, the anion ability of stabilizing the enzyme is in a decreased order of CH_3COO^-, $CF_3COO^- > Cl^-$, $Br^- > OTs^- > BF_4^-$. At first, we suspected that the polarity of the solvent might be a major factor for different enzyme activities. Therefore, we measured the wavelengths of maximum absorption of Nile Red dissolved in various solvents based on a literature method [42] and calculated their molar transition energies as shown in Table 2. The lower the molar transition energy is, the higher polarity a solvent has. These values were in agreement with previously reported data that most ILs polarities are close to those

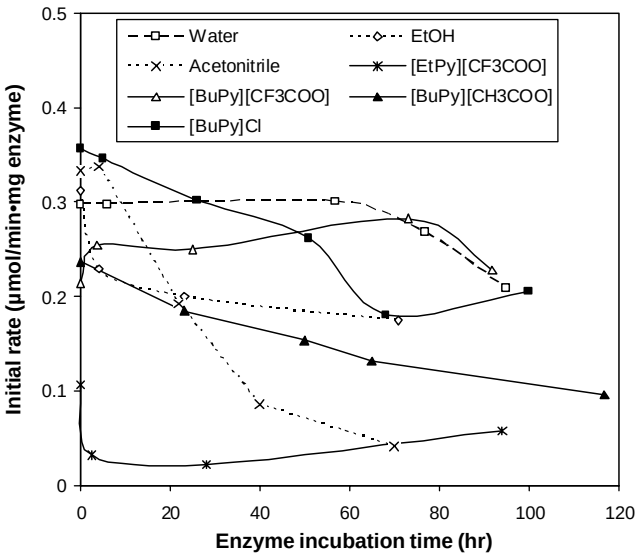


Fig. 3. Stability of protease P6 in various pyridinium based ionic media (0.7 M).

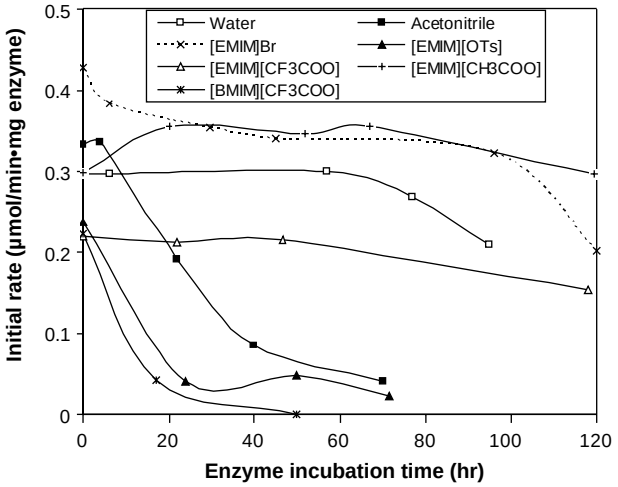


Fig. 4. Stability of protease P6 in various imidazolium based ionic media (0.7 M).

Table 2
Wavelengths of maximum absorption (λ_{max}) and molar transition energies (E_{NR}) for Nile Red dissolved in various solvents

Solvent	λ_{max} (nm)	E_{NR} (kJ mol^{-1})
Water	659	181.6
Methanol	549	218.0
Ethanol	547	218.8
Acetonitrile	533	224.6
[EtPy][BF_4]	561	213.4
[EtPy][CF_3COO]	566	211.5
[BuPy][BF_4]	562	213.0
[BuPy][CF_3COO]	566	211.5
[BuPy][CH_3COO]	560	213.8
[EMIM][BF_4]	591	202.5
[EMIM][CF_3COO]	549	218.0
[EMIM][CH_3COO]	573	208.9
[BMIM][BF_4]	545	219.6
[BMIM][CF_3COO]	552	216.9

Note. $E_{NR} = (hcN_A/\lambda_{max}) \times 10^6$, where h is the Planck's constant, c is the speed of light, N_A is Avogadro's number, and λ_{max} is the wavelength of maximum absorption (nm).

of lower alcohols [42,45] or formamide [46]. However, based on Table 2, the polarities of these ILs are not quite different. Therefore, the IL polarity could not entirely explain the differences of enzyme activities.

A plausible explanation is derived from the effect of ion kosmotropicity on the protein stability as discussed previously, i.e., kosmotropic anions stabilize proteins and chaotropic ones destabilize them. One of the most indicative parameters for quantifying the ion kosmotropicity is the viscosity *B*-coefficients [47]. The *B*-coefficients for CH_3COO^-, Cl^-, Br^-, and BF_4^- are 0.246, −0.005, −0.033, and −0.093, respectively (Fig. 1). A more positive *B*-value means a higher kosmotropicity. Therefore, CH_3COO^- is a kosmotrope; Cl^- is a borderline ion; Br^- is a weak chaotrope, and BF_4^- is a strong chaotrope. The *B*-coefficient

of CF_3COO^- is unknown, however, Hartmann [48] considered this anion as a weaker perturbant of water structure than CH_3COO^- but stronger than Cl^- based on the infrared spectral examinations of OH-stretching frequency shifts in HOD. This comparison was also confirmed by the NMR B'-coefficients [49]. Therefore, the anion kosmotropicity is in the decreasing order of $CH_3COO^- > CF_3COO^- > Cl^- > Br^- > BF_4^-$. This order is consistent with our series of the anion ability to stabilize the protease. Especially, due to the high kosmotropicity of CH_3COO^-, the enzyme exhibited a much higher stability in 0.7 M [EMIM][CH_3COO] than in pure water (Fig. 4). OTs^- is suspected to be a chaotrope because of its large size and single charge, even though its B-coefficient is unknown [9].

However, it still seems suspicious that the BF_4^- anion inhibited the protease activity immediately (1 min incubation) despite it is a strong chaotrope. We measured the pH values of 0.7 M BF_4^- based ILs and they were rather acidic (pH 3–4). This is probably because BF_4^- ions are not quite stable and may release HF in aqueous solutions. The low pH environment was undesirable for the enzyme. In addition, BF_4^- is a strong chaotrope. However, when the BF_4^- based ILs were maintained in buffers, this anion did not completely inhibit the enzyme activity as indicated in Table 1. Meanwhile, under the anhydrous condition, the enzyme might also stay active in BF_4^- based ILs [27,50,51] because these ILs did not dissociate into ions in the absence of water.

Two halide-based ILs (e.g., [BuPy]Cl and [EMIM]Br) did not seem to strongly destabilize the enzyme (Figs. 3 and 4). The possible reason is because Cl^- is a borderline ion and Br^- is a weak chaotrope, and they have relatively weak abilities of destabilizing proteins [52,53]. The significance of this finding is that impurities of halides in ILs may not considerably change the enzyme activity, especially when these halides are present in low concentrations.

3.3. Effect of cations on the enzyme stability

From Fig. 3, the protease maintained relatively high activities in $[BuPy]^+$ based ILs than in [EtPy][CF_3COO]. Similarly, from Fig. 4, higher enzyme stabilities were observed in $[EMIM]^+$ based ILs than in [BMIM][CF_3COO]. Based on these data, the cation ability of stabilizing a protease is in a decreasing order of $[EMIM]^+$, $[BuPy]^+ > [BMIM]^+ > [EtPy]^+$.

Therefore, IL cations also play an important role on the enzyme stability. Since chaotropic cations stabilize proteins and kosmotropic ones destabilize them, inhibition of enzyme activity was observed when only (non-ionic) chaotropes or kosmotropes were used in the enzymatic system [54]. It has been known [8] that larger tetraalkylammonium cations, such as $[n\text{-}Pr_4N]^+$ ($B = 0.916$), $[n\text{-}Bu_4N]^+$ ($B = 1.275$), and $[n\text{-}Pe_4N]^+$ (B unknown), are kosmotropes; smaller $[Et_4N]^+$ ($B = 0.385$) is a borderline ion and $[Me_4N]^+$ ($B = 0.123$) is a chaotrope. We calculated the B-coefficients of $[MePy]^+$, $[EtPy]^+$, and $[BuPy]^+$ as 0.144, 0.228, and 0.396 [9]. Comparing these B-values with those of tetraalkylammonium ions, $[MePy]^+$ and $[EtPy]^+$ are chaotropes while $[BuPy]^+$ is a borderline ion. The order of increasing kosmotropicity is $[MePy]^+ < [EtPy]^+ < [BuPy]^+$. All of these three cations could potentially stabilize the enzyme depending on their combinations with anions as well as the specific enzymatic application. The B-coefficients of imidazolium cations are not yet available, however, some preliminary thermodynamic results [55–57] implied that $[EMIM]^+$ is a chaotrope and $[BMIM]^+$ is a kosmotrope based on their interactions with water molecules. Therefore, the former cation stabilized the enzyme while the later cation destabilized it as illustrated in Fig. 4.

For different biological macromolecules, ions do not necessarily stabilize or destabilize them in the same kosmotropicity order [6,54,58]. By comparing different cations, it seemed that $[BuPy]^+$ and $[EMIM]^+$ could pair with kosmotropic anions to form more suitable chaotrope–kosmotrope environments for the protease to be active than $[EtPy]^+$ and $[BMIM]^+$ could. Therefore, the protease exhibited higher stabilities in $[BuPy]^+$ and $[EMIM]^+$ based ILs than in $[EtPy]^+$ and $[BMIM]^+$ based ILs. However, it must be aware that no matter which ions are present, if the ion concentration is too high, the enzyme could lose its activity due to the high ionic strength.

3.4. Stability of other enzymes in ILs

Fig. 5 illustrated the stability of alcalase (a protease) and PPL (a lipase) in 0.7 M solutions of $[BuPy][CF_3COO]$ and $[EMIM][CF_3COO]$. Overall, the enzyme activities were relatively stable in these ionic media (except alcalase in $[BuPy][CF_3COO]$), which confirmed that these two ILs are capable of stabilizing enzymes. Alcalase exhibited higher activity than PPL. A possible reason was that alcalase was water-soluble while PPL was insoluble (suspended in solutions). The increasing activity of PPL was probably due to a better dispersion of the enzyme in IL solutions over time.

4. Conclusions

The high enzyme stabilities were achieved in aqueous solutions through using ILs with a chaotrope (cation) — kosmotrope (anion) combination. Kosmotropic anions (such as CF_3COO^- and CH_3COO^-) stabilize the protease, while chaotropic ones destabilize it. The organic cations in ILs exhibited an opposite effect: chaotropic cations (usually small sizes) stabilize the protease, while kosmotropic ones (large sizes) destabilize it. The choices of cations and anions for a specific application should rely on the individual enzyme–medium–substrate relationship.

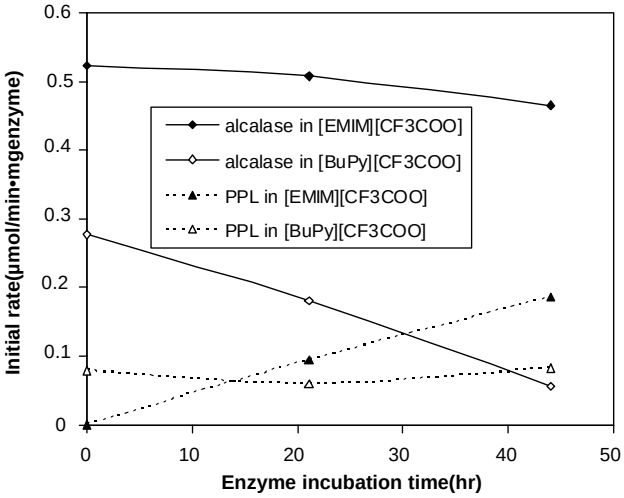


Fig. 5. Stability of alcalase and PPL in 0.7 M ILs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bioorg.2005.10.004.

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